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Formation and resolution of ankylosis under application of recombinant human bone morphogenetic protein-2 (rhBMP-2) to class III furcation defects in cats

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Objectives: Periodontal regeneration under application of bone morphogenetic protein (BMP) is compromised by ankylosis. Ankylosis disappearance following application of BMP has been observed in the case of a small defect, which might be beneficial change for periodontal regeneration. However, the histological observation of ankylosis disappearance has not been demonstrated in a large defect. The purpose of this present study was to confirm resolution of ankylosis during periodontal regeneration by recombinant human BMP-2 (rhBMP-2) applied to class III furcation defects.

Material and methods: Class III furcation defects were created in the premolars of six adult cats. The rhBMP-2 material, prepared by applying rhBMP-2 to a combination of polylactic acid–polygricolic copolymer and gelatin sponge (PGS; 0.33 μg rhBMP-2/mm^3 PGS) or control material containing only PGS, was implanted into each defect. The cats were killed at 3, 6 or 12 weeks after surgery and serial sections were prepared for histological and histometrical observation.

Results: Ankylosis was observed in some of the rhBMP-2/PGS group at 3 and 6 weeks, but not at 12 weeks. At 6 weeks, osteoclast-like cells were visible in the rhBMP-2/PGS group with ankylosis. Residual PGS was evident between the bone and root surface in the rhBMP-2/PGS group without ankylosis at 3 weeks.

Conclusions: Resolution of ankylosis by osteoclast-like cells possibly occurred under application of rhBMP-2. Residual PGS might play an important role in preventing ankylosis formation.

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Bone morphogenetic protein (BMP) has the ability to induce bone formation through multiple effect on bone homeostasis. Many studies have reported that recombinant human BMP-2 (rhBMP-2) and recombinant human osteogenic protein-1 (rhOP-1/BMP-7) promote regeneration of alveolar bone, cementum, and periodontal ligament in animal in which periodontal defect had been artificially created (1–11). It is therefore expected that BMPs will be used in future for periodontal regeneration therapy in humans.

For successful periodontal regeneration therapy, substantial regeneration of the alveolar bone and cementum regeneration and functional orientation of the periodontal ligament are necessary. Unfortunately, however, periodontal ligament regeneration in rodents and canines with periodontal defects that have been treated with rhBMP has sometimes been compromised by ankylosis (1, 2, 5, 6, 9, 10). Ankylosis disturbs new cementum formation and periodontal ligament growth and results in replacement of the periodontal ligament space with bony union to the root surface (2, 5, 9, 10).

On the other hand, King and Hughes (7) reported that the ankylosis disappeared after implantation of rhBMP-2 in experimentally created fenestration defects of rats. They found partial ankylosis at 10 days after implantation of rhBMP-2, but not at 35 days. Resolution of ankylosis may promote reestablishment of periodontal ligament space with periodontal ligament growth and restore periodontal ligament homeostasis (12). Therefore, the occurrence of ankylosis may not be a serious problem if it is completely resolved. However, disappearance of ankylosis might occur because of a small periodontal defect and ankylosis in the study by King and Hughes (7). Moreover, resolution of ankylosis had never been reported in cases of large periodontal defects such as horizontal defect and class III furcation defect. Therefore, it remains unclear whether the ankylosis can be reduced during periodontal regeneration by BMP applied to large defects.

The aim of this study was to confirm histologically and histometrically that ankylosis is resolved during periodontal regeneration by rhBMP-2 in class III furcation defects.

Material and methods

Animals

Six adult mongrel cats, all males weighing about 4.0 kg, were used in the experiment. This study was performed according to a protocol approved by the Laboratory Animal Care and Use Committee, Hokkaido University Graduate School of Dental Medicine.

Presurgical oral hygiene phase

The animals were anesthetized to undergo full-mouth scaling 1 month prior to surgery. All teeth were scaled and planed using hand and ultrasonic instrumentation. The anesthetics for the presurgical phase were chlorpromazine hydrochloride [Contomin®, 2.5 mg/kg intramuscularly (i.m.), Mitsubishi Pharma Corporation, Osaka, Japan) and ketamine hydrochloride (Ketalar®, 20 mg/kg i.m., Sankyo Co., Ltd, Tokyo, Japan). Swabbing with 0.2% chlorhexidine solution using a cotton-tipped applicator and supragingival debridement were performed in each animal once a week to maintain oral hygiene.

Implant materials

The rhBMP-2 was obtained from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). A combination of polylactic acid–polygricolic copolymer and gelatin sponge (PGS, Yamanouchi Pharmaceutical Co., Ltd) was used as the rhBMP-2 carrier. RhBMP-2 (solvent: LF6 buffer, 5 mM glutamic acid, 5 mM sodium chloride, 2.5% glycine, 0.5% sucrose, 0.01% Tween 80, pH 4.5) material, prepared by applying rhBMP-2 to PGS (4 μg rhBMP-2/2 mm × 2 mm × 3 mm PGS) was used as an implant material in the experiment site. The ratio of rhBMP-2 to PGS used was based on the results of our preliminary experiment. The PGS (5 mm × 3 mm × 1 mm) containing 0, 1, 5, or 10 μg rhBMP-2 was implanted on the distobuccal bony plate of each canine in the cats ($N = 4$). New bone formation was observed at 5 and 10 μg rhBMP-2/15 mm^3 PGS. We also found that 5 μg rhBMP-2/15 mm^3 PGS (0.33 mg rhBMP-2/mm^3 PGS) is a good combination ratio for promotion of bone formation. Control material containing only PGS in LF6 buffer was used as an implant material for the control site.

Surgical phase

Class III furcation bony defects were artificially created in the 3rd maxillary and 3rd and 4th mandibular premolars. Prior to surgery, the animals were pre-anesthetized with injection of chlorpromazine hydrochloride (Contomin®, 2.5 mg/kg i.m.) and anesthetized with ketamine hydrochloride (Ketalar®, 20 mg/kg i.m.). In addition, local anesthetic (Xylocaine®, lidocaine HCl 2% with 1/80000 epinephrine, Fujisawa Pharmaceutical Co., Ltd, Tokyo, Japan) was applied to the surgical site.

In each animal, the mucoperiosteal flaps on the buccal and lingual sides were elevated, and the class III furcation bony defect was created around the premolars by using a hand instrument, a diamond bur and a round steel bur. Following root planing, notches were made in the medial root surface and in the distal root surface at the bottom of the defect. The vertical distance between the bottom of the defect and the cement-enamel junction was about 3.5 mm. The experimental or control implant materials were placed to class III furcation bony defects. All the prepared class III furcation defects in maxillary and mandibular premolars received either one of two experimental conditions: rhBMP-2/PGS or PGS alone. Experimental conditions were rotated between defect sites in subsequent animals. The mucoperiosteal flaps were then replaced and sutured. Sutures were removed at 1 week after surgery. Swabbing with 0.2% chlorhexidine solution with a cotton-tipped applicator and supragingival debridement were performed in each animal once a week.

Seven premolars with short tooth roots and narrow furcation areas were revealed during surgical procedure. Finally, 15 teeth were used for the experiment site and 14 for the control site. In the rhBMP-2/PGS group, one 3rd maxillary and two 3rd and two 4th mandibular premolars (five teeth) were analyzed at 3, 6 and 12 weeks. In the PGS alone group, one 3rd maxillary and two 3rd and two 4th mandibular premolars (five teeth) were analyzed at 3 and 12 weeks, and two 3rd and two

4th mandibular premolars (four teeth) at 6 weeks (Table 1).

Histological procedures

The cats were killed at 3, 6 or 12 weeks after surgery. Block sections were obtained and immediately fixed in 10% buffered formalin. Then the sections were decalcified, trimmed, dehydrated, and embedded in paraffin. Serial sections of 7 μm thickness were cut in a mesio-distal plane throughout the entire buccal-lingual extension of the tooth. Every 14th section was stained with hematoxylin and eosin to enable observations at intervals of 100 μm. From these sections, it was possible to identify the section representing the central aspect of defect.

The most central section for each premolar tooth root was identified by the size of the root canal. The two buccal and two lingual sections were those adjacent to the most central sections. Then, a total of five sections representing the buccal (two sections), central (one section) and lingual (two sections) aspect of the defects were selected for each tooth. The sections were analyzed histologically, and the images of all sections and a micrometer were taken using a light microscope interfaced to a computer-based image software (Photoshop® 4.0, Adobe Systems Inc., San Jose, CA, USA). The following measurements at each section were performed by the micrometer on a computer.

Table 1. Number of teeth analyzed and frequency of teeth with ankylosis

	Group					
	PGS alone			rhBMP-2/PGS		
Weeks	P^3	P_3	P_4	P^3	P_3	P_4
3	0/1	0/2	0/2	0/1	2/2	0/2
6	0/0	0/2	0/2	0/1	2/2	1/2
12	0/1	0/2	0/2	0/1	0/2	0/2
Total	0/2	0/6	0/6	0/3	4/6	1/6

P^3, 3rd maxillary premolar; P_3, 3rd mandibular premolar; P_4, 4th mandibular premolar; rhBMP-2, recombinant human bone morphogenetic protein-2; PGS, polylactic acid–polygricolic copolymer and gelatin sponge.

1 Defect height (mm), i.e. the distance between the bottom line of the defect and the fornix of the furcation (Fig. 1).
2 New bone formation (mm), i.e. the mean value of mesial new bone and distal new bone measurements (the distance between the bottom of the defect and the coronal termination of new bone formation were measured along the mesial new bone and distal new bone root surface) (Fig. 1).
3 Ankylosis (mm), i.e. frequency and linear length of the ankylotic bone.

Data analysis

Defect height and new bone in the five selected sections were averaged in each tooth. The means and standard deviation of defect height and new bone were calculated using a tooth as one unit for each experimental condition (rhBMP-2/PGS group and PGS alone group) at each observation interval (3, 6, and 12 weeks). Differences between the rhBMP-2/PGS group and the PGS alone group were analyzed using the Mann–Whitney *U*-test. Statistical significant was determined considering an alpha level of 0.05.

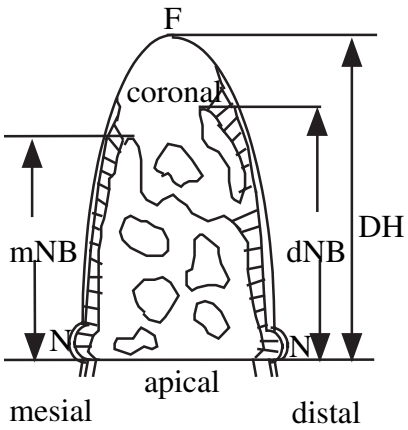

Fig. 1. Schematic furcation defect. N, the notch added root surface; F, fornix of furcation; DH, defect height; dNB, distance between the defect's bottom and the coronal termination of new bone formation were measured along the distal root surface; mNB, distance between the defect's bottom and the coronal termination of new bone formation were measured along the medial root surface.

Results

Histological observation

Table 1 shows the frequency of each premolar with ankylosis. In the rhBMP-2/PGS group, ankylosis occurred in two of the five teeth at 3 weeks and in three of the five teeth at 6 weeks. No ankylosis was seen at 12 weeks after surgery in the rhBMP-2/PGS group. However, ankylosis was not observed in the PGS group during the whole study period.

At 3 weeks after surgery, new bone formation in the PGS alone group was limited (Fig. 2a). Bone regeneration was obviously greater in the rhBMP-2/PGS group than in the PGS alone group (Figs 2a–c). However, ankylosis was observed in two of the five teeth for the rhBMP-2/PGS group (Table 1). Residual PGS was visible within the coronal area of defect in the rhBMP-2/PGS group (Fig. 2b and c). In the teeth in the rhBMP-2/PGS group that showed no ankylosis, newly formed bone was evident along the root surface (Fig. 2b). PGS and fibrous connective tissue were observed around new bone and the root surface (Fig. 2b). In the teeth in the rhBMP-2/PGS group that showed ankylosis (Figs 2c and d), newly formed bone was also visible. Osteoclast-like cells were not observed around the area of the ankylosis (Fig. 2d).

At 6 weeks after surgery, PGS had disappeared in both groups. In the rhBMP-2/PGS group, new bone was observed at the coronal aspects of the defects (Figs 3a and b), and three teeth had ankylosis (Table 1). Ankylosis was observed in two of two 3rd mandibular premolars and one of two 4th mandibular premolars (Table 1). Broad ankylosis at the coronal site was observed in the two 3rd mandibular premolars (Fig. 3a) and narrow ankylosis was in the 4th mandibular premolar (Fig. 3b). A space resembling the periodontal ligament space was observed at an apical site of ankylotic union (Fig. 3a). Resolution of ankylosis was observed in all teeth with ankylosis (Figs 3c and d). Osteoclast-like cells were observed around the area of the ankylosis, and part of the ankylotic

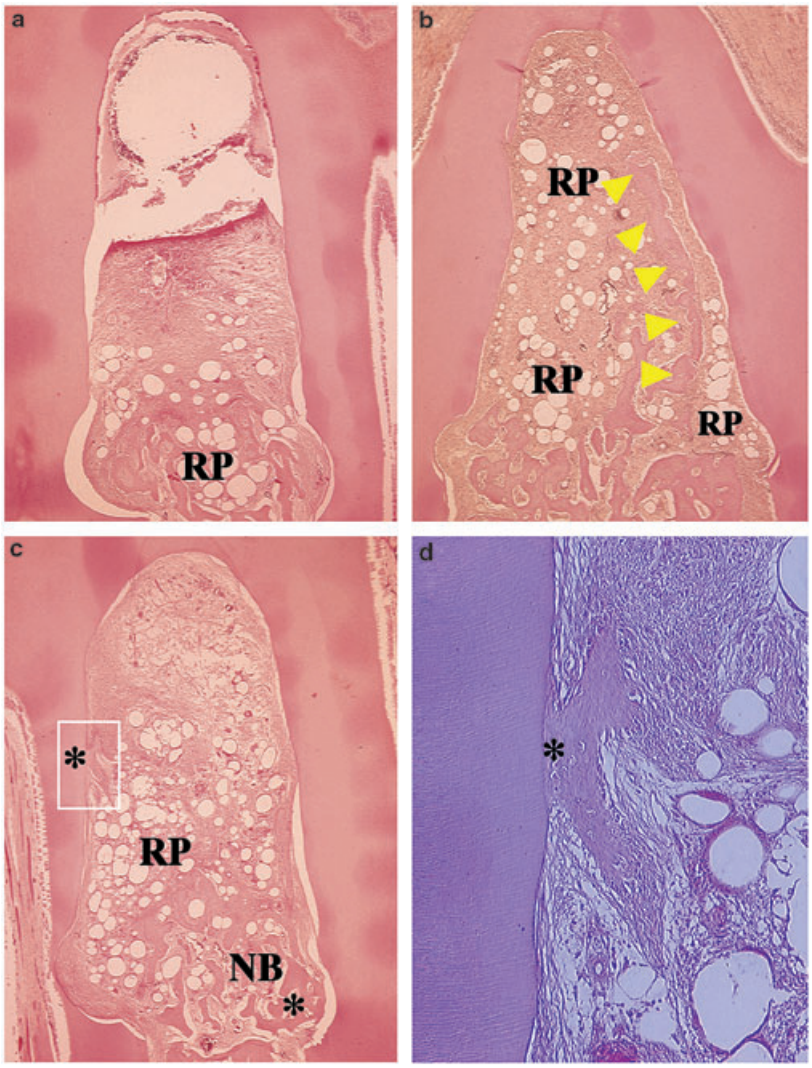

Fig. 2. Photomicrographs of defects implanted polylactic acid–polygricolic copolymer and gelatin sponge (PGS) alone (a) and recombinant human bone morphogenetic protein-2 (rhBMP-2)/PGS (b, c) at 3 weeks after surgery. Residual PGS (RP) was observed in both groups (a–c). New bone (arrowheads) was evident along the root surface and residual PGS (RP) was observed between new bone and the root surface (b). Ankylosis (*) was observed (c). High-power view of the area in C showing ankylosis (d).

bony union had been resorbed (Figs 3c and d). New cementum formed continuously beyond the notch was also observed at apical site of ankylosis (Fig. 3c).

At 12 weeks after surgery, new bone formation was limited and down growth of the junctional epithelium was observed in the PGS alone group. Almost complete healing of the defect occurred in the rhBMP-2/PGS group, with new bone formation, new cementum formation and periodontal ligament growth (Fig. 4a). No ankylosis was observed (Table 1). Root resorption lacunae were covered with new cementum and osteoclast-like cells were not observed on the root surface in the rhBMP-2/PGS group (Fig. 4b). Functionally oriented collagen fibers were inserted into the new cementum (Fig. 4b).

Histometric analysis

Table 2 shows the results of histometric analysis. No significant difference was formed between initial bone defect height (DH) in the PGS alone and rhBMP-2/PGS groups. New bone height at 3, 6 and 12 weeks after surgery were 0.70 ± 0.13, 1.20 ± 0.13 and 1.74 ± 0.31 mm, respectively, in the PGS alone group and 1.69 ± 0.35 mm, 2.40 ± 0.26 and 2.74 ± 0.29 mm, respectively, in the rhBMP-2/PGS group. New bone height was significantly greater in the sites to which rhBMP-2 had been applied than in the control sites at any of the postoperative observation times ($P < 0.05$). Linear lengths of ankylosis at 3 and 6 weeks after surgery were 0.22 ± 0.15 mm and 0.41 ± 0.23 mm, respectively.

Discussion

The objective of this study was to confirm the reduction or disappearance of ankylosis following application of rhBMP-2 to class III furcation defects. Artificial class III furcation defects to which rhBMP-2/PGS or PGS alone had been applied were monitored over a 3-, 6- and 12-week healing period. As a result, ankylosis was evident at 3 and 6 weeks after surgery and the length of ankyloitic bony union at 6 weeks was longer than at 3 weeks in the rhBMP-2/PGS group. At 6 weeks after surgery, resolution of ankylosis with osteoclast-like cells was observed. Root resorption was observed around the area of ankylosis, and new cementum was formed on the root surface. A space resembling the periodontal ligament space was observed between new bone and the tooth root at an apical site of the area of ankylosis. At 12 weeks after surgery, ankylosis was not evident and almost complete regeneration of periodontal tissue was observed. Although the same teeth were not monitored in this study, it may be possible that ankylosis had increased until about 6 weeks after surgery and that the resolution or disappearance of ankylosis by osteoclast-like cells had then occurred.

One reason for prevention of ankylosis or promotion of ankylosis disappearance may be a certain extent of occlusal loading. Masticatory function has been shown to stimulate the repair process to resolve ankylosis with the development of periodontal ligament space after systemic administration of

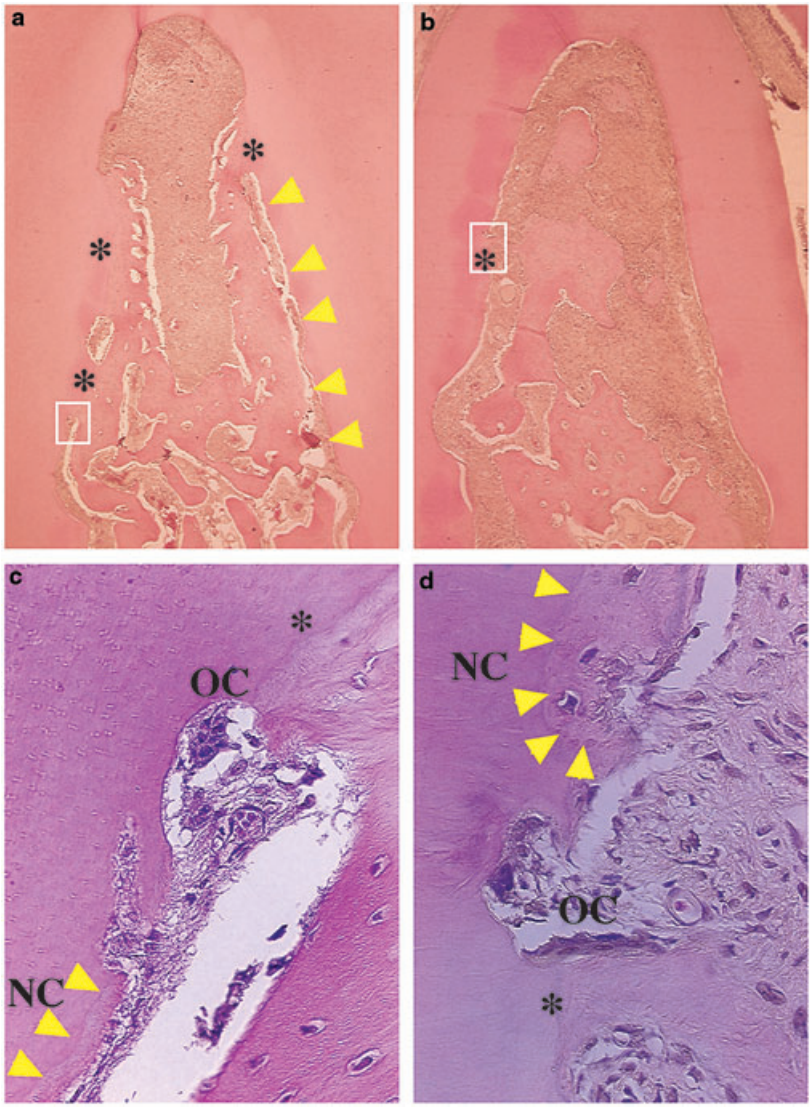

Fig. 3. Photomicrographs of defects implanted recombinant human bone morphogenetic protein-2 (rhBMP-2)/polylactic acid–polygricolic copolymer and gelatin sponge (PGS) with ankylosis (*) at 6 weeks after surgery in the 3rd mandibular premolar (a, c) and 4th mandibular premolar (b, d). A space resembling the periodontal ligament space (arrowheads) was observed at an apical site of ankylotic union (a). High-power view of the area in A (c) showing presence of osteoclast-like cell (OC). New cementum (arrowheads) formed continuously was observed at the apical site of osteoclast-like cell (c). High-power view of the area in B (d) showing presence of osteoclast-like cell. New cementum (NC) formed on root resorption laqunae around the ankylotic area (arrowheads).

1-hydoroxyethylidene-1,1-bisphosphonate in periodontal ligament resulting in an ankylosis in mice (12). ElDeeb and Andreasen (13) investigated the effect of occlusion on healing of periodontium after surgical injury in rats without using any regeneration materials. A significant narrowing of the periodontal ligament width was observed in the non-occluding teeth, whereas occluding teeth did not show significant narrowing of the periodontal ligament width.

Our finding of ankylosis also supports this hypothesis. In the present study, ankylosis was totally observed in five teeth (two at 3 weeks and three at 6 weeks, Table 1) for the rhBMP-2/PGS group. Four of the five teeth with ankylosis were the mandibular 3rd premolars. There is generally no occlusal contact in the 3rd premolar area of the cats because of the small size of the opposing tooth. In the 4th premolar area, however, there is clear occlusal tooth contact at mastication. It is conceivable that functional stimulation applied to the 4th premolar is stronger than that applied to the 3rd premolar and the substantial growth of periodontal ligament-like tissue was promoted by functional stimulation in 4th premolars. It may be possible that differences of occlusal loading between the 3rd and 4th premolar effect ankylosis formation.

Another factor contributing to prevention of ankylosis may be duration of occlusal loading. In the case of occurrence of ankylosis, resolution of ankylosis by osteoclast-like cells was observed at 6 weeks after surgery. In the 4th mandibular premolar, ankylosis was observed at 6 weeks after surgery and the ankylosis was very narrow. At 12 weeks after surgery, ankylosis was not observed. Following the resolution of ankylosis by osteoclast-like cells, disappearance of ankylosis might have occurred between 6 and 12 weeks in the present study. Disappearance of ankylosis or transient ankylosis was also observed in previous studies (7, 14). King and Hughes (7) found partial ankylosis at 10 days but not at 35 days after implantation of rhBMP-2 in fenestration defects created in rats. Based on these findings, continuous application of proper occlusal loading until 6 weeks at the latest using temporary prosthetics might be helpful in order to prevent ankylosis formation.

There were some methodological limitations in the present study that should be considered when interpreting the results. Because the occlusal forces were not determined, it is unclear that the occlusal forces actually affected the results. However, it is hard to measure the occlusal forces in the cat mouth. As mentioned previously, same teeth were not monitored throughout the study period. Consequently, the histological sections from different animals were analyzed at each week. Therefore, it might not be proper to suggest that a previously ankylosed site became 'de-ankylosed' later. Preliminarily, we used the radiographs to monitor the

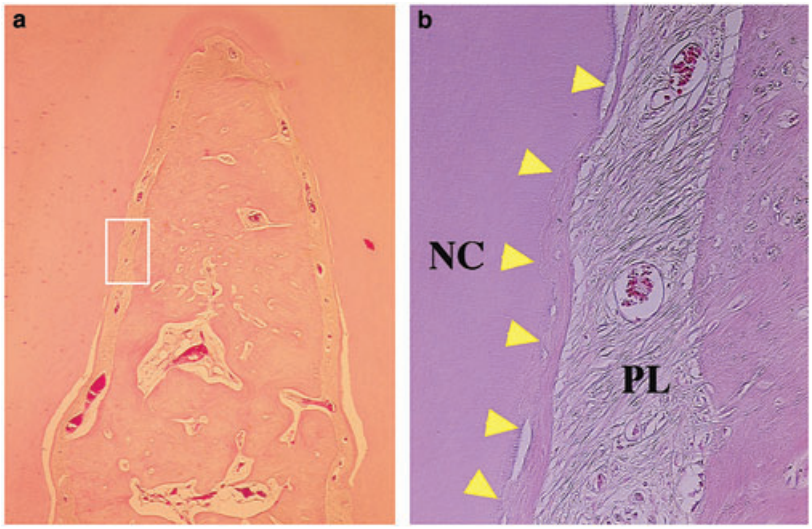

Fig. 4. Photomicrographs of defects implanted recombinant human bone morphogenetic protein-2 (rhBMP-2)/polylactic acid–polygricolic copolymer and gelatin sponge (PGS) (a) at 12 weeks after surgery. High-power view of area in a (b) showing functionally oriented periodontal ligament (PL) insert into the new cementum (NC).

Table 2. Results of histometric measurements (mean ± SD in mm)

Week	Group	*n*	Defect height	New bone formation	Ankylosis
3	PGS alone	5	3.35 ± 0.21	0.70 ± 0.13	0
	rhBMP-2/PGS	5	3.16 ± 0.06	1.69 ± 0.35*	0.22 ± 0.15
6	PGS alone	4	3.16 ± 0.13	1.20 ± 0.13	0
	rhBMP-2/PGS	5	3.32 ± 0.09	2.40 ± 0.26*	0.41 ± 0.23
12	PGS alone	5	3.45 ± 0.18	1.74 ± 0.31	0
	rhBMP-2/PGS	5	3.45 ± 0.12	2.74 ± 0.29*	0

*Significantly greater than the PGS alone group by Mann–Whitney *U*-test ($P < 0.05$).
rhBMP-2, recombinant human bone morphogenetic protein-2; PGS, polylactic acid–polygricolic copolymer and gelatin sponge.

same site during the whole experiment and to estimate the amount of ankylosis. However, the radiographs could not clearly indicate the change of periodontium. A well-designed experiment with longer duration is required for accurate determination of change of ankylotic union.

We selected the sponge-type of PGS (a combination of polylactic acid–polygricolic copolymer and gelatin sponge) as a carrier of rhBMP-2 according to the study of Kinoshita *et al.* (4). They reported that bone and cementum regeneration was significantly increased in the rhBMP-2-treated group using PGS as a carrier than those in the control group using PGS alone. They also mentioned that the granules-type PGS were difficult to adapt to the root surface with horizontal circumferential bone loss, and the sponge-type PGS was suitable for periodontal reconstructive surgery.

Residual PGS might play an important role in periodontal regeneration in the early stage. In the present study, residual PGS was not evident near the root surface of the teeth in the rhBMP-2/PGS group with ankylosis at 3 weeks after surgery, whereas residual PGS was observed between newly formed bone and root surface in the area without ankylosis at the same experimental period. This finding shows that the decomposition of PGS had started during the 3 weeks after surgery, which resulted in the ankylosis formation. For the prevention of BMP-2-induced ankylosis, development of a carrier with the characteristics of slow decomposition is required. Modification of the ratio of polylactic acid–polygricolic copolymer to gelatin or usage of other material might induce the delay of PGS decomposition.

In summary, resorption of ankylosis by osteoclast-like cells was observed under application of rhBMP-2. Occlusal loading and residual PGS between bone and root surface might play an important role in prevention of ankylosis formation during periodontal regeneration.

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